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Studies on the Correlation of Brain Diazo Coupling Reaction Levels and the Activity of Para Substituted Phenols in Experimental Poliomyelitis

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STUDIES ON THE CORRELATION OF BRAIN DIAZO COUPLING REACTION LEVELS AND THE ACTIVITY OF PARA SUBSTITUTED PHENOLS IN EXPERIMENTAL POLIOMYELITIS

by

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APPROVAL SHEET

The thesis submitted by Pierre-Georges Roy has been read and approved by three members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

July 29, 1954
Date

Charles D. Proctor
Signature of Adviser

LIFE

Pierre-Georges Roy was born in Levis, Province of Quebec, Canada on January 20, 1926. After finishing his elementary school training he attended the Levis College of the Laval University of Quebec from 1937 to 1946. In the latter year he received the degree of Bachelor of Arts from that institution. In September, 1947, he undertook studies in pharmacy at the School of Pharmacy of Laval University from which school he was graduated with the degree of Bachelor of Science in 1951. During the same year he was admitted to the Graduate School of Loyola University of Chicago, beginning his studies there in September, 1951.

He was awarded a three year fellowship from the Provincial Government of Quebec in order to undertake his graduate studies.

ACKNOWLEDGEMENT

The author is deeply grateful to Doctor Charles D. Proctor, who, during the course of the research done on this problem, was his very kind and helpful advisor. Throughout this research work, his real scientific mind and his wonderful collaboration allowed the author to achieve the purpose of this thesis.

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CHAPTER I

INTRODUCTION

Research conducted by workers in this department over a number of years (Proctor and Byrd, 1947-) has revealed that three para substituted phenols exert a chemotherapeutic effect against the Lansing strain of the virus of Poliomyelitis in the mouse. The three compounds are para-hydroxy-benzoic acid, (N-(2-thiazolyl)-phenol sulfonamide (commonly called "Darvisul"), and (N-(2-pyrimidyl)-phenol sulfonamide. Their work has also demonstrated a difference in the degree to which these compounds are effective in producing this chemotherapeutic protection.

In view of the fact that the virus of Poliomyelitis is a neurotropic virus found predominantly in the central nervous system it was felt that in all probability any effect of these compounds on that virus would have to be exerted in the central nervous system. It is well known that chemical substitutions on aromatic nuclei can alter the physico-chemical properties of the parent compound and in many instances such alterations have affected the organ distribution properties of a given compound in animals. A pertinent example of this effect is seen in the class of aminobenzene sulfonamides where the relatively high distribution of the compound sulfadiazine in the brain is

markedly evident in contrast to relatively lower distributions of the other members of this class of compounds in the same organ. (Worthy, 1946). Since three compounds with varying degrees of effectiveness against experimental infections caused by a given neurotropic virus and with varied para substitutions to a common phenolic group were available, it was felt that a study of the distribution of these compounds in the brain following their administration would be of interest. It was reasoned that such a study might yield data which could be used in clarifying some of the reasons for the variation of the in vivo effect on the virus which has been observed in the case of these three compounds.

With this view point in mind the work carried out in this thesis has been undertaken with a three-fold purpose. This three-fold purpose is as follows: a. to set up a method for the determination of para-hydroxy-benzoic acid, (N-(2-thiazolyl)-phenol sulfonamide, and (N-(2-pyrimidyl)-phenol sulfonamide as diazo coupled compounds in the brain tissue of the mouse; b. to ascertain whether or not change in the phenol concentration of the mouse brain, as determined by diazo coupling reaction, occurs following administration of these compounds; c. to ascertain the correlation of such concentrations with the effect of these compounds against the Lansing strain of the virus of Poliomyelitis in the mouse.

CHAPTER II

SURVEY OF THE LITERATURE

A. THE CHEMOTHERAPEUTIC EFFECT OF SUBSTITUTED PHENOLS AGAINST EXPERIMENTAL POLIOMYELITIS IN THE MOUSE

A survey of the literature by the author has failed to reveal any published research which has been undertaken with the purpose of comparing the effects of para-hydroxybenzoic acid, (N-(2-thiazolyl)-phenol sulfonamide, and (N-(2-pyrimidyl)-phenol sulfonamide on the course of the experimental infection of the Lansing strain of the virus of Poliomyelitis in mice. To the author's knowledge the unpublished work done in this department (Proctor and Byrd, 1947-) represents the only such comparison yet attempted. In the course of their work in this field data has resulted which allows for qualitative and quantitative comparison of the effects of these compounds against this experimental virus infection in this animal. The experiments in their series which allow for the most direct comparison of the relative degree of effectiveness of these three compounds under the conditions being considered have been summarized in Table I. (The code letters designating the three drugs in this table are: NSD for (N-(2-pyrimidyl)-phenol sulfonamide, NST for (N-(2-thiazolyl)-phenol sulfonamide, and PHBA for para-hydroxybenzoic acid, respectively. These code letters will be used as such throughout the rest of

this thesis). The experiments presented in the table adequately establish a daily threshold protective dose, the effect of raising such doses, and the effect of such doses on survival rate over a long observation period. The only relative variables involved between experiments were the drugs administered, and, in the case of the PHBA, the dose of drug given. Scrutiny of Table I reveals that the daily threshold effective dose of HSD and HST are the same (1gm./Kg.) while that of PHBA (1.65 gm./Kg.) is higher than either HSD or HST. While the threshold effective doses of HSD and HST are the same, the effect of these two drugs on thirty day survival in the infected mouse is different, the HSD being superior to the HST in this respect. It would appear that PHBA, at its effective daily dose, is just as effective in exerting protection against the virus as is HST, though the dose of the former required for this activity is greater. On a basis of these observations from the work of Proctor and Byrd (1947-) the author has arranged the three substituted phenols in the following order of potency with respect to their in vivo protective effect against the virus in the mouse:

Greatest ---------- Least
HSD HST PHBA

In the case of HST research by workers other than Proctor and Byrd has been undertaken in order to establish the effect of this compound against experimental virus infections in the mouse. The first report on such work which the author has been able to locate is the study made by Sanders and his co-workers (1948) on the effect of HST against the Columbia Murine SK virus in

TABLE I

COMPARISON OF CHEMOTHERAPEUTIC EFFECT OF SUBSTITUTED PHENOLS AGAINST EXPERIMENTAL POLIOMYELITIS IN THE MOUSE *
(Proctor and Eyrd, 1947-)

Compound	Daily Threshold Effective Dose	Effect of Increase in Daily Dose Above Threshold Effective Dose	Effect of Threshold Effective Dose on Thirty Day Survival	Treated	Controls
HSD	1 gm./Kg.	none	expt. 1	16/30	0/30
			expt. 2	17/30	2/30
HST	1 gm./Kg.	none	expt. 1	7/30	0/30
			expt. 2	9/30	2/30
PHBA	1.65 gm./Kg.	none	expt. 1	10/30	0/30

* All drugs were administered orally from tragacanth suspensions of equal tragacanth concentration. All controls received equivalent volumes of tragacanth suspension without drug. Drug administration began 24 hours post-intracerebral innoculation of the virus (with 0.03 ml. of a virus dilution of 0.5×10^{-4}) and was administered once per day for nine additional days in each case. The LD50 of the virus was 1.0×10^{-3}. In the case of the 30 day survival experiments numerators indicate survivors, denominators indicate the total number of animals innoculated.

the mouse. These workers reported a chemotherapeutic protection exerted by HST against this virus in the mouse. In the next year Francis and Brown (1949) reported that HST failed to protect against this virus in the mouse. LoCrippo and his associates (1949) reported that the same compound failed to exert a protective effect against the Columbia Murine SK virus and other viruses, including the Lansing strain of the virus of poliomyelitis.

Several cogent observations can be made on these reports of the chemotherapeutic effect of para substituted phenols against poliomyelitis infections in the mouse. In the case of the first two reports on the effect of HST against the Columbia Murine SK virus, projection of an analogy between these two distinctly different viruses, is distinctly uncertain. In the case of the work done by the LoCrippo group (1949) the research reported does not supply sufficient evidence to reach a justifiable conclusion relative to the activity of HST against the Lansing strain of the virus of poliomyelitis in the mouse. In the single unrepeated experiment carried out by these workers using HST against this virus strain only eight animals were used per experimental variable, with one dose level of the drug tested. Proctor and Byrd (1947-) have ascertained that this compound does afford some protection against this virus in the mouse and that this can be demonstrated if proper attention is given to magnitude of dosage, length of dosing period, adequacy of the number of animals for statistical evaluation, etc.

In view of the above mentioned limitations inherent in the published reports which the author has been able to locate to date the criteria for

comparative evaluation of the chemotherapeutic effects of the para substituted phenols included in this study against experimental poliomyelitis infections in the mouse has been restricted to the results obtained by Proctor and Byrd.

B. METHODS FOR THE DETERMINATION OF PHENOLS IN BIOLOGICAL MATERIAL

There is widespread literature available concerned with the determination of phenols in biological material. The majority of these reports deal with this determination in body fluids such as blood and urine. It is beyond the purpose of this thesis to include an exhaustive review of the voluminous literature in this field. This is especially so in view of the adequate review articles which have been written on the subject from time to time. (Barac, 1935 a & b) (Diechman and Schafer, 1942) (Bray, et al, 1952). The general methods which have enjoyed extended use include those based on iodometric titration (Messinger and Vortmann, 1889), colorimetric estimation with phosphotungstic-phosphomolybdic acid (Folin and Denis, 1915), colorimetric estimation using Millon's reagent (Haas & Schlesinger, 1924), and colorimetric estimation by means of chromophores produced when the phenol is coupled with diazo reagents such as diazotised para-nitroaniline (Theis and Benedict, 1924) or diazotised sulfanilic acid (Hanke and Koessler, 1922). Many modifications of these methods have been made. The review articles by Barac (1935 a & b) are especially valuable in that he reached tentative conclusions from the reports which existed up to the time of his work and then proceeded to test his conclusions by running comparative quantification and recovery experiments. In

his opinion the methods using diazo reagents were superior to all of the other general methods advanced up to his time. Subsequent reviewers (Diechmann and Schafer, 1942) (Bray, et al, 1952) have agreed with this conclusion. While methods using diazo reagents for phenol determination are not wholly specific for phenol, Barac demonstrated that methods using them were more specific than the other techniques and were more efficient in effecting recovery of phenol added to biological material than were the other methods. (Barac, 1935 a & b). The author searched the literature both independently and inclusive of the literature quoted by the reviewers and as a result of the search came to a tentative conclusion which was essentially the same as that of the reviewers cited. For this reason the diazo reagent methods were selected for study in relationship to their possible application to the determination of the substituted phenols included in this research problem.

C. STUDIES ON THE DISTRIBUTION OF PHENOLS TO THE BRAIN

An extensive survey of the literature by the author has failed to reveal any studies made on the distribution of phenols to the brains of mice following administration of such compounds to these animals. No literature on the normal levels of phenols in the mouse brain was encountered.

Traces of phenolic bodies in the brain tissue of essentially normal human beings were found by Diechmann and Schafer (1942). These workers used diazotized para-nitroaniline reagent for the determination of the phenol. Smith (1933) determined that appreciable quantities of phenol were distributed in the brains of phenol poisoned rabbits. He used the Folin-Denis method (1915)

to determine phenol in steam distillates of the brain tissue. From in vitro and in vivo studies Smith concluded that very little, if any, conjugation of phenols occurs in the brain. Deichmann (1944) during an investigation of the toxicity of phenol reached the conclusion that the tissues of normal rabbits and rats contain insignificant traces of free and conjugated phenols. In his studies he ascertained that the largest amount of ingested phenol was recovered from the organs and tissues of rabbits dying in 15 minutes from toxic doses of phenol, while the smallest amounts of phenol were recovered from these sites in the case of animals living for six hours, the longest period of survival in his study. It is also interesting to note that in studies on surviving rabbits given phenol conducted by Deichmann the distribution of free phenol in the brain seems to occur in such a manner as to yield peak concentration levels with apparent ebb and flow of the distribution of the free, unconjugated phenol

CHAPTER III

METHODS AND MATERIALS

1. Comparative Study of Methods for the Determination of the Substituted Phenols in the Brain: As has been stated in the previous chapter it was decided that the general method selected for study in term of its application to the determination of the substituted phenols used in this problem was the colorimetric method utilising diazo reagents for development of chromophore compounds from phenols. Theis and Benedict (1924) had used diazotized para-nitroaniline as a reagent for this purpose in determining the phenolic content of the blood. Hanke and Koessler (1922) had reported the use of diazotized sulfanilic acid for the determination of phenol. It was decided that both of these methods should be investigated for possible application to the determination of HST, HSD AND PHBA in the brain of the mouse.

In the attempts made to adapt the original Theis and Benedict method to a determination of the three substituted phenols difficulty was encountered either in determinations attempted on the pure compounds or on deproteinized extracts made of the compounds after their prior addition to brain tissue. In the case of the author's early attempts at the adaptation of this method to the determination of HST, HSD and PHBA it was ascertained that color developed by the method in solutions containing the increasing increments of drug concentra-

tion was no different than that developed in the reagent blank. This was true in the case of either determinations attempted on the pure compounds or in the instances where the compounds were present in deproteinised extracts of the brain tissue. Realising that the stability of diazotised para-nitroaniline can be a function of the pH of the solution in which it is present (in some systems at alkaline pH it is converted to nitrosamine) (Feiser and Feiser, 1950) it was decided to investigate the effect of varying the pH of the final reaction mixture of the Theis-Benedict method.

The variations in pH of the final reaction mixture were accomplished by effecting many different modifications of the original method. Some of these included use of various buffers to yield varied final pH values, use of varied amounts and varied concentrations of sodium hydroxide solution and use of varied amounts and varied concentrations of sodium carbonate solution in order to attempt location of an optimal system for color development proportional to concentration. As a result of these experiments it was ascertained that optimal color development was obtained when 0.8 ml. of twenty per cent sodium carbonate was added to the final Theis-Benedict reaction mixture to yield pH values uniformly within the range of 7.5 to 8.5. Under these conditions pure solutions of the substituted phenols gave results which indicated a fairly reasonable relationship between concentration and optical density.

However, when this modified method was tried out on brain or liver tissue to which solutions of the phenolic derivatives had been added erratic results were obtained. Frequently optical density measurements were made

uncertain by the presence of turbid precipitates in the solutions available for spectrophotometric measurement. There were many possible situations which could engender such an impass to application of the method. Some of these are: incomplete deproteinisation, co-precipitation of incompletely precipitated protein and the chromophores, precipitation of the chromophores, precipitation of unreacted phenolic compounds, precipitation of reagents, etc. A large number of modifications designed to alleviate these and similar possibilities were designed and tested. Protein precipitants, including tungstic acid, phosphotungstic acid, trichloroacetic acid, silicotungstic acid, lead acetate and ammonium sulfate were tried singly, and, in some cases, in combination, at varied concentrations and with and without water bath heating of the sample. Various agents designed to minimise precipitation of one or the other of the substances possibly responsible for the turbidity observed were tried. Agents tested for this purpose included ethyl alcohol, alkaline ethyl alcohol, propylene glycol, gum tragacanth, gum acacia, acetone and pyridine. Conditions of concentration, point of addition in the method, heating, etc., were varied considerably in the case of each one of these modifying agents. None of the changes which have been described (in a general way) produced results which could be repeated with constancy and reliability in the presence of tissue (brain and liver). In like manner addition of stoichiometric or excess amounts of ammonium sulfamate to destroy excess nitrous acid in the diazo reagent failed to improve the results. Because of these difficulties encountered in both the use of the Theis-Benedict method and the author's attempted modifica-

tions of it, it was felt that attention should be given to the diazotised sulfanilic acid reagent method of Hanke and Koessler (1922).

Early in the preliminary investigation carried out on the application of this method to the determination of the three phenolic derivatives it became apparent that it had several advantages over the diazotised para-nitroaniline reagent method. Reagent blanks were uniformly lower in optical density over the whole wave length range of the colored spectrum than were those obtained in the case of the Theis-Benedict reagents. No turbidity was present in either cases where the method was applied to pure solutions of the drugs or in the cases where drugs had been added to brain followed by deproteinization and subsequent application of the method. Advantages in time saved and accuracy achieved were realised by several modifications made by the author and by rejection of several modifications which had been suggested previously by others. The author ascertained that more efficient deproteinisation of the brain tissue was effected using trichloracetic acid and silicotungstic acid in combination than with any of the other protein precipitants which he investigated including the phosphotungstic acid used by Hanke and Koessler (1919) in the original method. He ascertained that his final modification of the method yielded colors with the three compounds under study which gave stable optical density readings within a time range of fifteen to twenty minutes after development if read against a reagent blank. Preliminary work also indicated that the suggestion of Macpherson (1942) that alkaline alcohol be used to stabilise the color for longer than twenty minutes could not be applied to the method in the

low concentration ranges demanded by the work at hand. Such a modification uniformly lowered the optical density per unit concentration or substituted phenol. A saving in time and increased control over reagent stability was realised by diazotizing the sulfanilic acid at room temperature directly in each individual determination solution through direct addition of sodium nitrite solution to the reaction mixture rather than resorting to use of a diazo reagent made up by daily diazotization of a sulfanilic acid stock solution. Because preliminary studies indicated the possible utility of the Hanke-Koessler method or a suitable modification of it, more extensive studies on the application of it to the desired determinations of the three substituted phenols were undertaken.

2. Study of the Method Adopted for Determination of the Substituted Phenols in Brain Tissue: Following the preliminary comparative studies of methods outlined in the preceding section a method was obtained which seemed suitable for application to the determination of the substituted phenols in brain tissue. An outline of the procedure finally adopted is given below in two sections which list reagents used and procedure employed in such determinations.

Reagents

1. Ten per cent trichloroacetic acid: 50 grams of analytical reagent grade trichloroacetic acid are dissolved in and diluted up to 500 ml. of distilled water. (stable for several weeks).

2. Ten per cent silicotungstic acid: 10 grams of analytical reagent grade silicotungstic acid are dissolved in and diluted to 100 ml. with distilled water. (stable for several weeks).

3. One per cent sulfanilic acid: 2.5 grams of analytical reagent grade sulfanilic acid are dissolved in and diluted up to 250 ml. with a solution of sulfuric acid which contains one ml. of analytical reagent grade concentrated sulfuric acid dissolved in and made up to 250 ml. with distilled water. (stable for several weeks)

4. One per cent sodium nitrite: 1 gram of analytical reagent grade sodium nitrite is dissolved in distilled water and diluted up to 100 ml. with distilled water. (stable for three days)

5. Twenty per cent sodium carbonate: 20 grams of analytical grade anhydrous sodium carbonate are dissolved in and diluted up to 100 ml. with previously boiled and cooled distilled water. (stable if kept reasonably covered)

6. Concentrated standard solutions of HSD, HST and PHBA: 250mgm. of the desired compound are weighed out accurately on the analytical balance and added to a 125 ml. erlenmeyer flask. Two hundred mgm. of sodium carbonate and 50 ml. of distilled water are added. The flask is heated gently until all of the compound is in solution. After allowing the flask to cool to room temperature the contents are transferred to a 100 ml. volumetric flask along with two 10 ml. distilled water washings of the erlenmeyer flask. The solution is finally diluted to the 100 ml. mark in the volumetric flask with distilled water. Such solutions contain 2,500 mcg./ml. concentrations of the given drug.

7. Dilute standard solutions of HSD, HST AND PHBA: 1 ml. of the desired concentrated standard solution (above) is added by 1 ml. volumetric pipette to a 100 ml. volumetric flask and diluted up to 100 ml. with distilled water. Such solutions contain 25 mcg./ml. of the given drug.

Procedure

1. One lobe of the mouse brain was removed immediately following sacrifice of the animal (by a blow on the head) and weighed on a Roller-Smith balance to the nearest milligram.

2. The weighed lobe was placed in a mortar with about 0.2 gm. of chemically pure washed sand and 1 ml. each of 10 per cent trichloroacetic acid and 10 per cent silicotungstic acid added in that order. The brain tissue was ground in this mixture with the pestle for several minutes.

3. The protein precipitated brain mixture obtained was then washed into a ten ml. centrifuge tube with two 2 ml. portions of distilled water and the tube centrifuged for 20 minutes at 1,600 X g.

4. Following the centrifugation the supernatant liquid was decanted off into a test tube.

5. The following reagents were added to the decanted solution in order: (a) 2 ml. of 1 per cent sulfanilic acid; (b) 1 ml. of one per cent sodium nitrite.

6. The tube was allowed to stand for two minutes and 1 ml. of 20 per cent sodium carbonate was added with shaking.

7. The contents of the tubes were transferred to cuvettes and were read in the Coleman spectrophotometer at exactly fifteen minutes following the addition of the sodium carbonate. All readings were taken against a reagent blank set at zero per cent transmission at a wave length of 530.

The procedure just outlined was tested in several ways to establish quantification, specificity and recovery properties of the method. In order to establish the relationship between drug concentration and optical density, different aliquots of the dilute standard solutions of the respective drugs were incorporated into the procedure (with appropriate adjustment for volumetric differences) in place of brain tissue and plots of optical density vs. drug concentration obtained at wave length 530. (This wave length had been selected because there was adequate absorption at that point with the greatest difference between solutions containing drugs and the reagent blank being evident when both were read against distilled water set at zero per cent transmission—see figure 2). The relationship between drug concentration and optical density under conditions of the method for all three drugs is illustrated in figure 1. It is obvious that a straight line relationship holds in all three instances.

Recovery of substituted phenols added to the brain tissue was carried out using paired lobes of brain from the same mouse in order to avoid errors

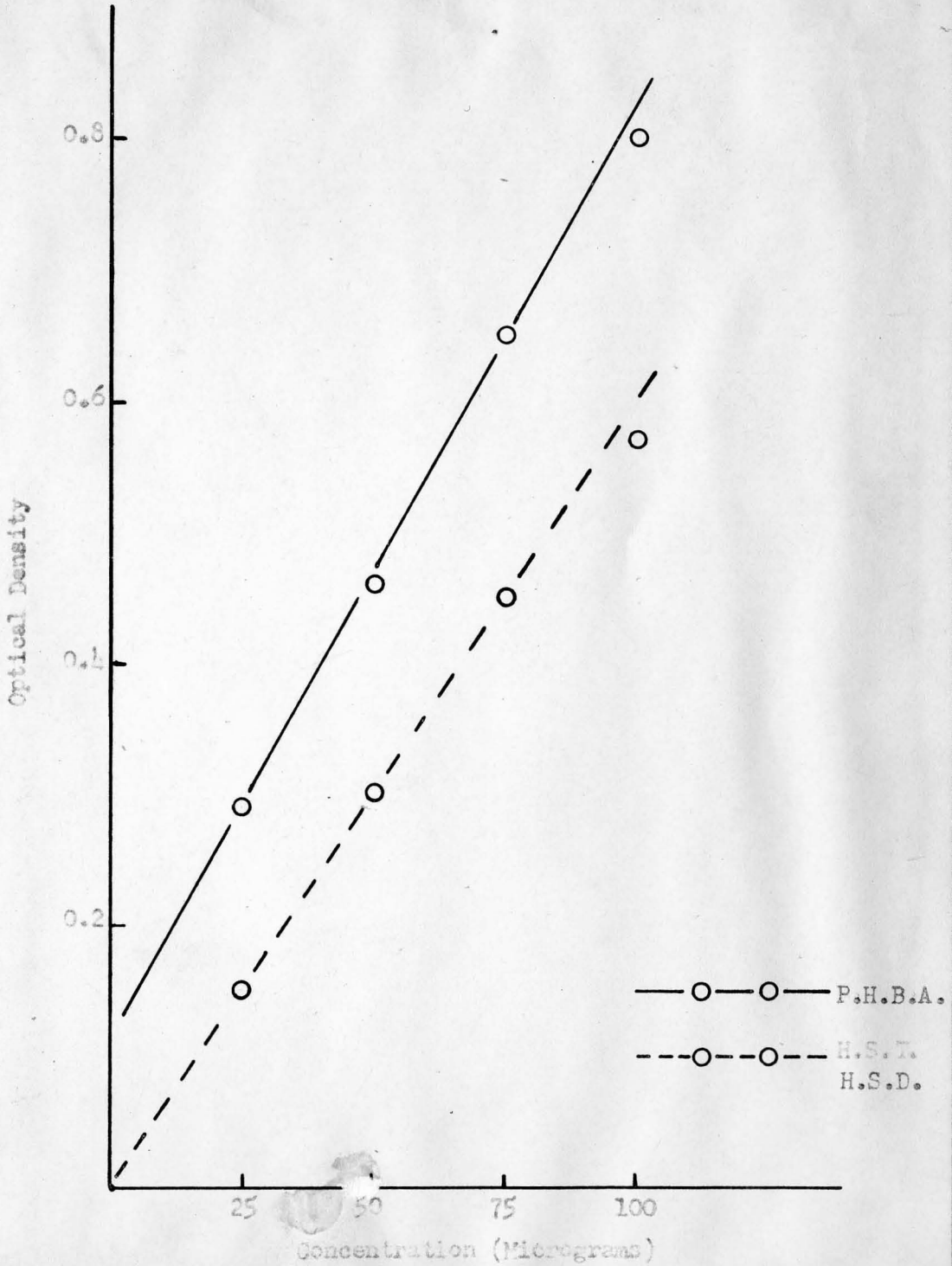

Figure 1. Optical Density Vs Drug Concentration of P.H.B.A., H.S.D. and H.S.T.

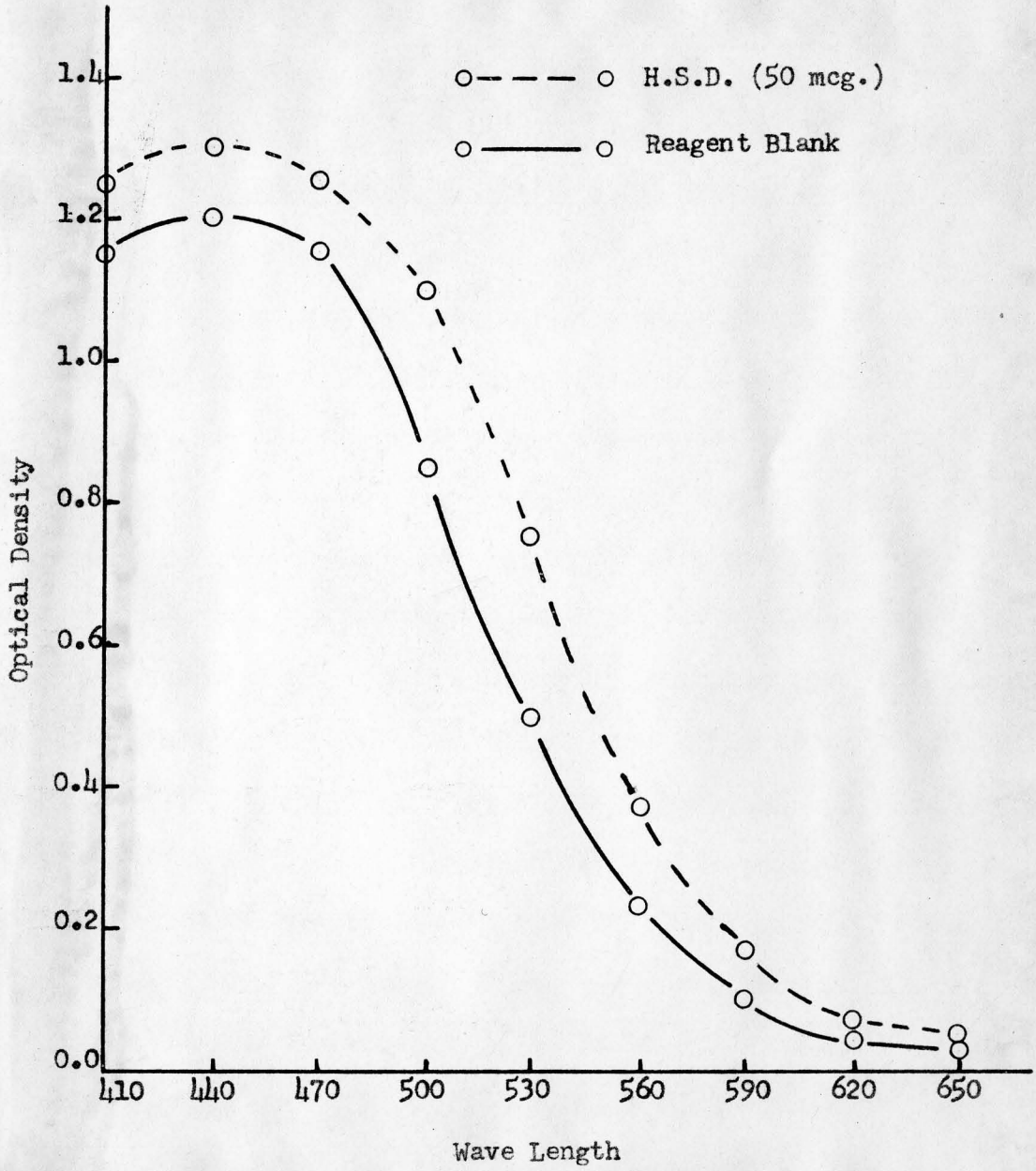

Figure 2. Absorption Curve of Reagent Blank and Drug Solution (H.S.D. (50 mcg.)

due to biological variation in the diazo reaction values found in different mice. Preliminary experiments had shown that very little if any inter-lobe variation in diazo reaction values was obtained in comparative analyses conducted on the individual lobes of brain taken from the same mouse. In these experiments the brain of the mouse was removed and the lobes separated and weighed. One of them was incorporated into the analytical procedure as the control and the other was incorporated into the procedure along with a definite aliquot of the dilute drug standard solution under study. The analytical procedure was continued and the difference between the two determinations taken as an indication of the degree to which recovery of the added drug had been effected. The recovery range achieved with drug additions between 25 to 100 mcg. were as follows: PHBA, 74-80 per cent; HET, 80-83 per cent; HED, 75-92 per cent. While there is an obvious loss of compound the recovery values obtained for these compounds using this method are at least equal to, and, in some cases, superior to those obtained by other investigators using protein precipitation to isolate phenol from tissues (Marenzi, 1932; Smith, 1933; Deichmann and Scott, 1939). They are equal to recoveries obtained by workers who extracted phenol from biological material with organic solvents, for subsequent determination (Deichmann and Schafer, 1942; Schmidt, et al, 1937; Schmidt, 1942, 1943; De Meio, 1948). In view of the relative insolubility in organic solvents of the compounds under study, the degree of recovery effected under conditions of protein precipitation was considered fortunate.

It was ascertained in experiments designed to test the specificity

of the method that imidazole compounds such as histidine and histamine and naturally occurring phenols such as tyrosine couple with diazotized sulfanilic acid under the conditions of the determination for the substituted phenols as outlined. These findings are in agreement with the findings of Barac (1935 a & b). Because of this interference from naturally occurring compounds it was felt that the application of the method as modified would yield concentration levels of diazo equivalents representative of the sum total of imidazole and phenol bodies naturally present in the case of brains from control animals and the sum total of imidazole, natural phenolic compounds and administered phenols distributed in the brain in the case of drug treated animals.

3. Materials and Procedures Used in the Study of the Distribution of the Substituted Phenols to the Brain: In order to closely approximate the animal species and strain used by Proctor and Byrd (1947-) in their virus experiments young Swiss albino mice of the Webster strain, weighing 16-20 grams, obtained from the same animal colony (Harlan Small Animal Industries, Cumberland, Indiana) used as a supply source by those workers, were used throughout these experiments. These stock animals were maintained on a uniform diet consisting of Purina chow and water ad libitum both at the source of supply and in the hands of the author. Several days were allowed for the animals to acclimatize to the new environment following the arrival of each shipment before they were used in any phase of the study.

The drugs used were from the same sources used by Proctor and Byrd. The PHBA was an Eastman Kodak Company highest purity grade chemical. It

melted at 215° C. HST was synthesised according to the method of Hultquist (1950). It melted at 230° C. HSD was synthesised according to the method of Proctor (1951). It melted at 232° C. Suspensions of these drugs for oral administration were prepared by incorporating them into one per cent tragacanth suspensions. HST and HSD were used at a 5 per cent concentration while PHBA was used at an 0.2h per cent concentration in these suspensions. The higher concentration used in the latter case was employed in order to circumvent excessive administered volume variations created by the relatively higher dose levels used in the case of this drug. In those experiments designed to test the influence of the drug vehicle aequeous one per cent tragacanth suspensions containing no drug were used.

Experiments of two general types were designed to test the distribution of the substituted phenols in the brain of the mouse. All of these experiments were set up so as to utilise the oral route of drug administration by stomach tube. This had been used exclusively by Proctor and Byrd (19h7 -) in their experiments on the effects of these compounds on experimental poliomyelitis infections in mice. One group of experiments was planned in which optimal conditions of absorption from the gastrointestinal tract and optimal distribution from the circulation across the blood-brain barrier might obtain. Theoretically such a state would occur under conditions in which the contents of the gastrointestinal tract and the circulatory transport of absorbed nutritional metabolites were minimised at the time of oral drug administration. To approximate this state experiments were carried out in which the drugs were

administered to animals (with free access to water) subjected to varied periods of fasting time pre- and post-drug administration. Control animals allowed water ad libitum but timed in an analogous manner with reference to fasting period time were run for comparison with the drug treated animals. It became apparent early in the work that the period of fasting could significantly affect the control diazo reaction level mean in the mouse so that strict analogy between drug treated and control animals in relationship to fasting period time was maintained in the case of the fasting experiments. While such experiments do not exactly parallel the conditions in the Proctor-Byrd chemotherapeutic experiments (their mice had free access to food) none-the-less it was felt that valuable fundamental information of the compounds in the brain under optimal conditions would be obtained from studies of this type. The other group of experiments, analogous to the Byrd-Proctor experimental conditions with the exception that the virus infection was not present, were run on groups of animals with free access to food and to which a daily oral dose of drug was administered. Suitable control animals were run for comparison with the drug treated animals in this experimental group. These two general experimental procedures are described in more detail in the discussion which follows.

In order to establish control values for the diazo reaction levels in mice in the fasting experiments, groups of 12-18 mice were used for each of the fasting periods which served as a reference for comparison of drug treated groups of animals. (Smaller groups of six animals were used at different intermediate fasting time periods in order to establish the degree of regularity

present in the diazo reaction level fluctuation observed with increase in fasting time.) These mice were transferred to a cage devoid of food at a given time and then at the desired interval of time following this transfer they were sacrificed by a blow on the head, the brain removed, and one lobe of the brain submitted to the analytical procedure outlined in the previous section. Mean values for fasting intervals of 3, 3½, 6, 9, 15, 18 and 24 hours were ascertained in this manner. Of these intervals only the mean values for the 3, 3½, 9, 15 and 24 hour periods were used as reference controls for drug treated animals in fasting experiments. In the case of drug treated fasting animals the drugs were administered at a convenient time interval following subjection of the animals to fasting. This was done in such a manner so as to allow for sacrifice of the animals at a time when the desired time interval of exposure to the drug coincided with one of the established control intervals of fasting time. For example, if it was desired that the effect of a drug exposure time of seven hours should be studied the animals would be subjected to fasting, the drug administered after two hours of fasting, and the animals sacrificed at the ninth hour after fasting had begun. The methods of sacrifice and analysis used in the case of drug treated animals were identical with those used in the case of the controls. In this manner mean diazo reaction values were obtained for the brain in the case of mice treated with 1 gm./Kg. of HSD and 1 gm./Kg. of HST at 1, 7, 13 and 22 hours post-administration of the drugs. Similar values were obtained for PHBA in the case of mice treated with 1 gm./Kg. and 1.65 gm./Kg. of this drug at ½, 1, 1½, 7 and 22 hours post-

administration of the drugs. Similar values were obtained for PHBA in the case of mice treated with 1 gm./Kg. and 1.65 gm./Kg. of this drug at ½, 1, 1½, 7 and 22 hours post-administration of the drug.

In the non-fasting experiments carried out on groups of 22-36 animals in the experimental (drug treated) groups and on 18 control animals (untreated) food intake was measured by daily weighing of the animal cage food supply cups which were stocked with ground Purina chow. No significant differences were noted in comparisons of average daily food intake by control and experimental animals in any case. Drug treated animals received a fixed oral dose of a given drug under study at a fixed time in the morning every twenty-four hours. At successive daily intervals subgroups of the drug treated animals (10-12 animals per subgroup) were sacrificed and the mean diaso reaction levels determined by analyses made on their brains. Sacrifice of subgroups of drug treated animals at successive 24 hour intervals during the experimental run was accomplished in each instance twenty-four hours after the last desired drug administration to the subgroup. Under these conditions it was possible to ascertain whether or not the compound being studied maintained any rise in the mean brain diaso reactim value during intervals of drug administration analogous to daily dose of 1.65 gm./Kg., HSD at a dose of 1 gm./Kg. per day, and HST at a dose of 1 gm./Kg. per day were administered were carried out under these conditions. Twenty-four hour maintenance levels of PHBA and HST were ascertained for 48 hour period and the analogous maintenance level for HSD was established over a 72 hour period.

Control of the drug suspending vehicle was accomplished by comparing mean diazo reaction levels of mice treated with 1 per cent aequeous tragacanth suspension in volumes equivalent to the administered volumes of the drug suspensions with the mean diazo reaction levels of untreated mice. Twelve animals were used per experimental variable in these experiments and under none of the experimental conditions outlined above did the vehicle treated groups of animals yield mean diazo reaction levels which were significantly different from those of untreated controls.

All differences in means obtained when control animals were compared with drug treated or vehicle treated animals have been analyzed for statistical significance by means of t^2 analysis of the data. The statistical constants obtained are listed in the appendix of the thesis.

CHAPTER IV

RESULTS AND DISCUSSION

The results obtained from experiments designed to demonstrate whether or not variation in control mean brain diazo reaction equivalents occur with respect to period of fasting time are illustrated in figure 3. That the fluctuation shown is probably real has been indicated by statistical analysis demonstrating statistical significance for the differences between means which make up the peaks and troughs of this curve. Such findings point out the soundness of using controls fasted analogously to fasted drug treated animals in the experiments planned to establish optimum conditions for distribution of the drugs in the brain.

While the fasting experiments do not parallel in the normal mouse the conditions maintained by Proctor and Byrd in their virus infected mice with free access to food it is still felt that hypothetical correlation of the two sets of results is possible if the lack of parallelism is kept in mind. The results of those experiments have been presented in graphic form in figures 4, 5, and 6. Figures 4 and 5 indicate that administration of the substituted phenols brought about definite rises in the mean brain diazo reaction equivalents over the corresponding control values regardless of the time elapsing

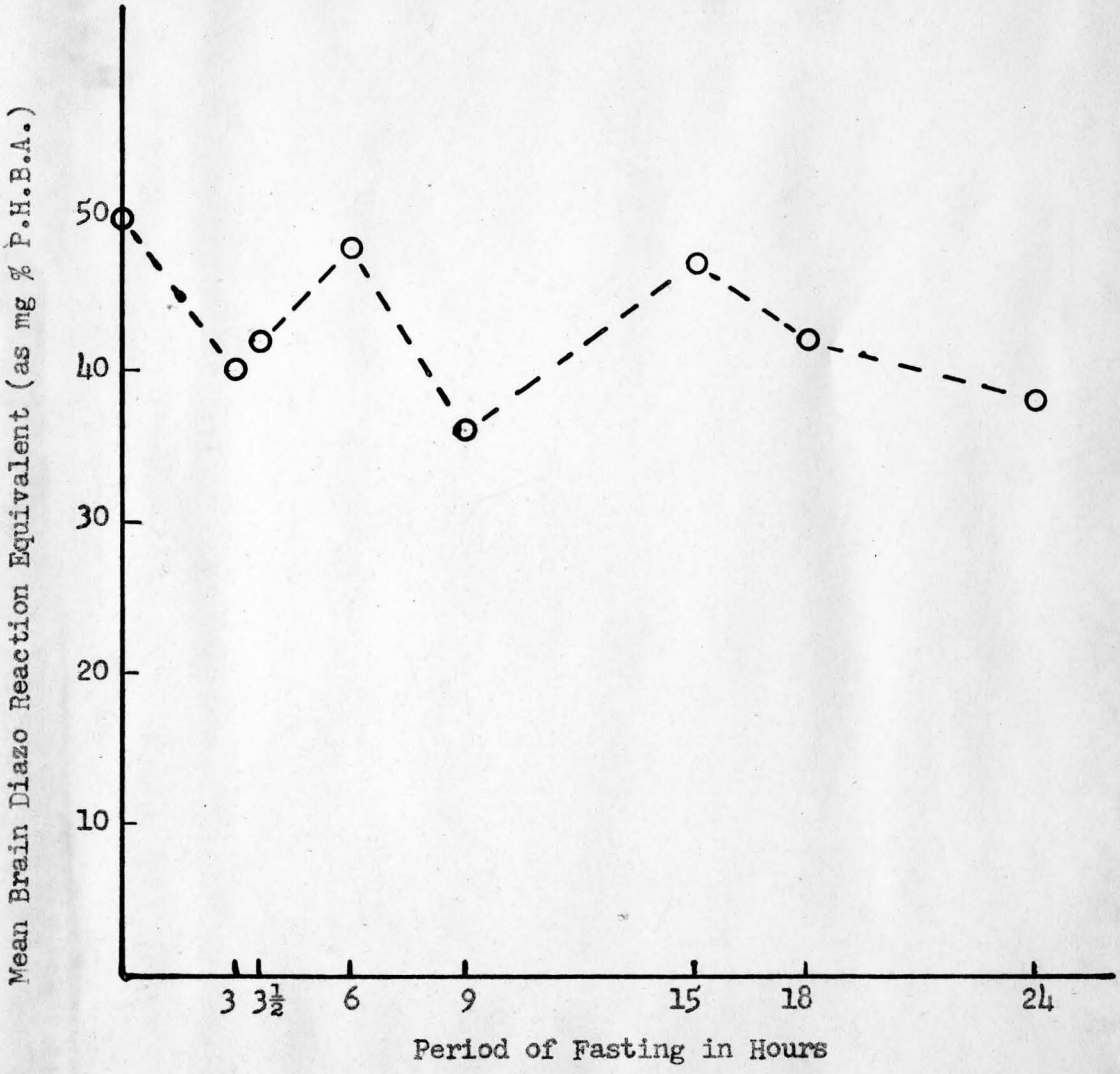

Figure 3. Variations in Mean Diazo Reaction Brain Levels with Fasting Period Time.

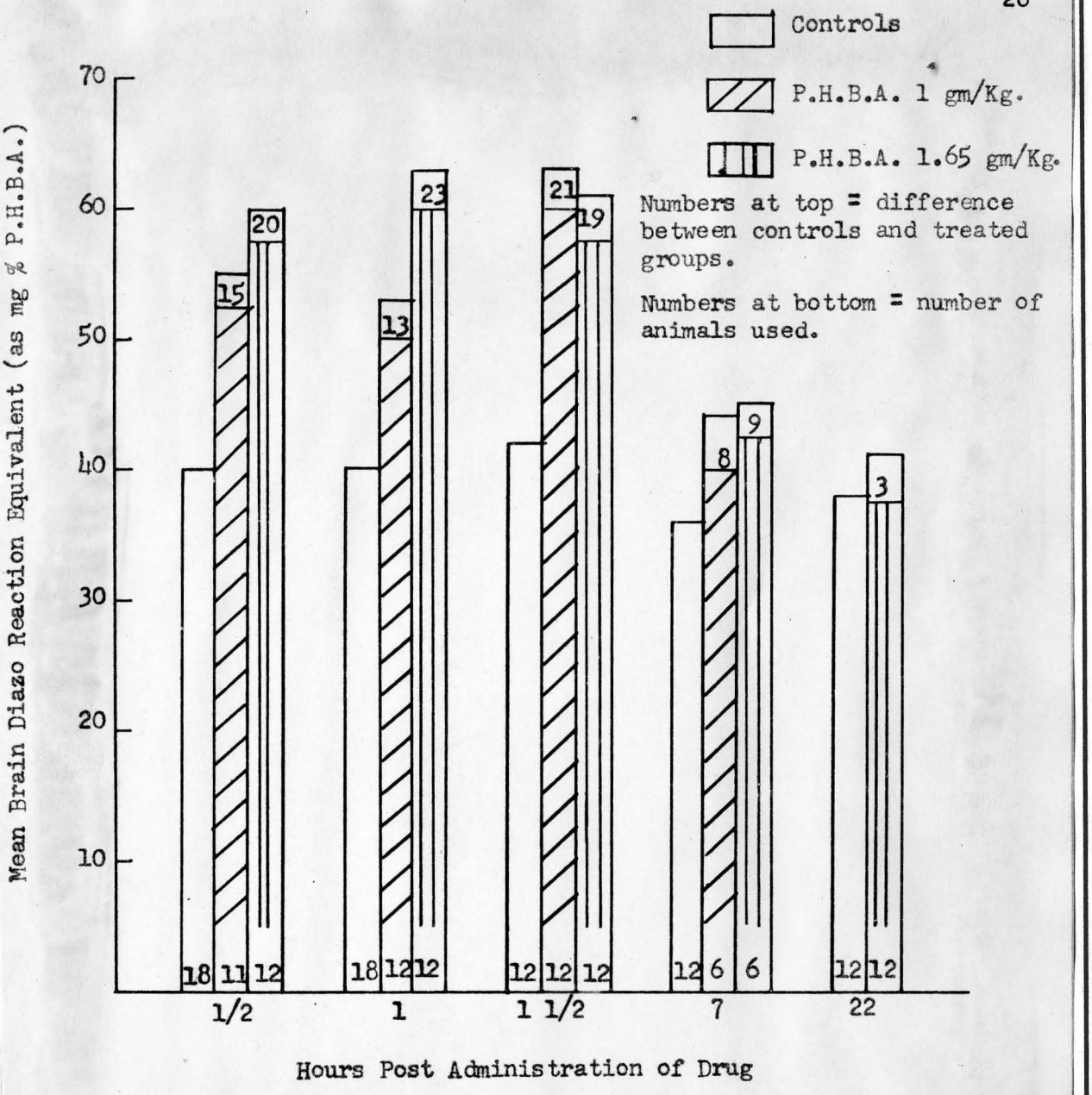

Figure 4. Effect of P.H.B.A. on Mean Brain Diazo Reaction Levels in Fasting Animals

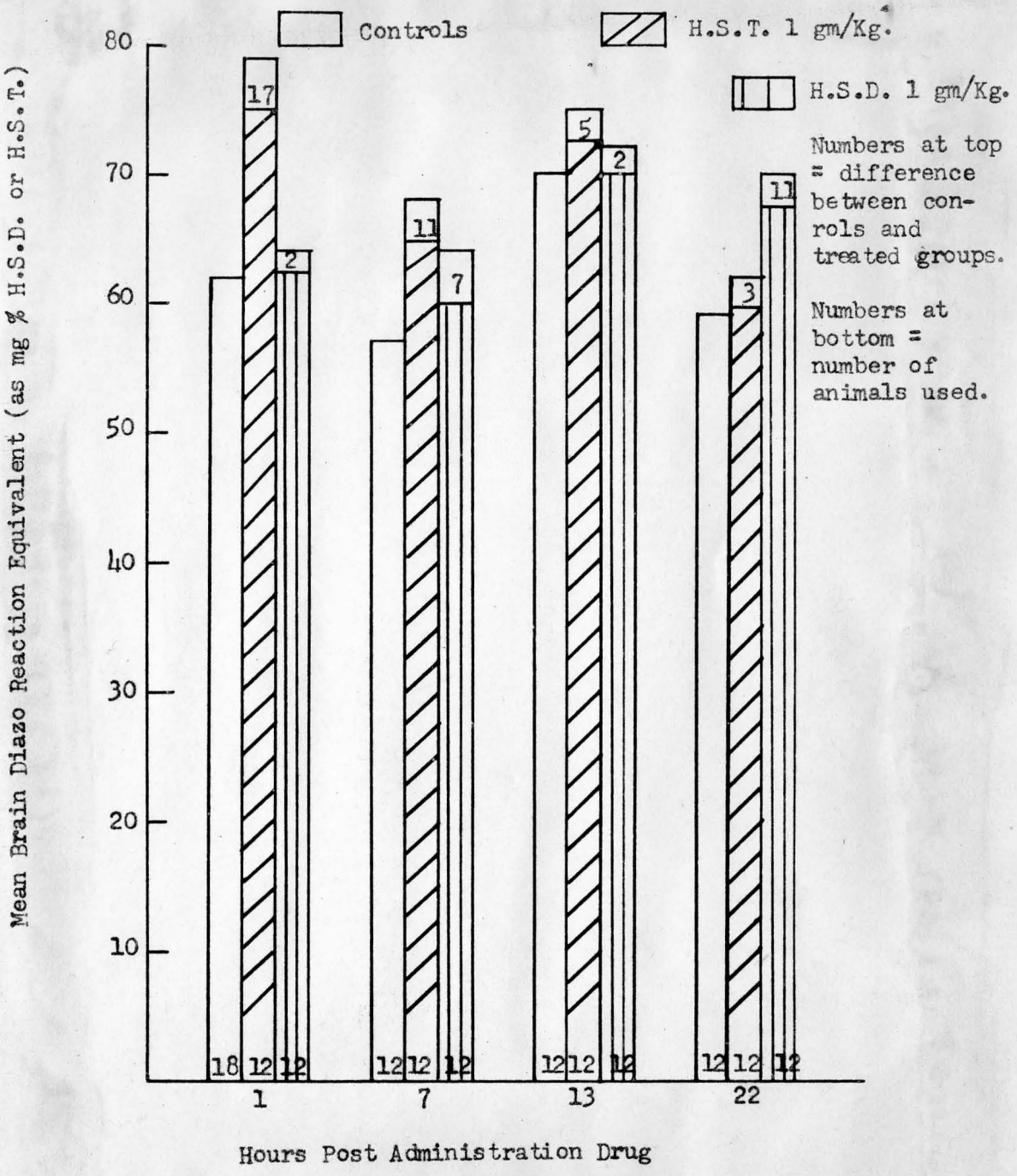

Figure 5. Effect of H.S.D. and H.S.T. on Mean Brain Diazo Reaction Levels in Fasting Animals.

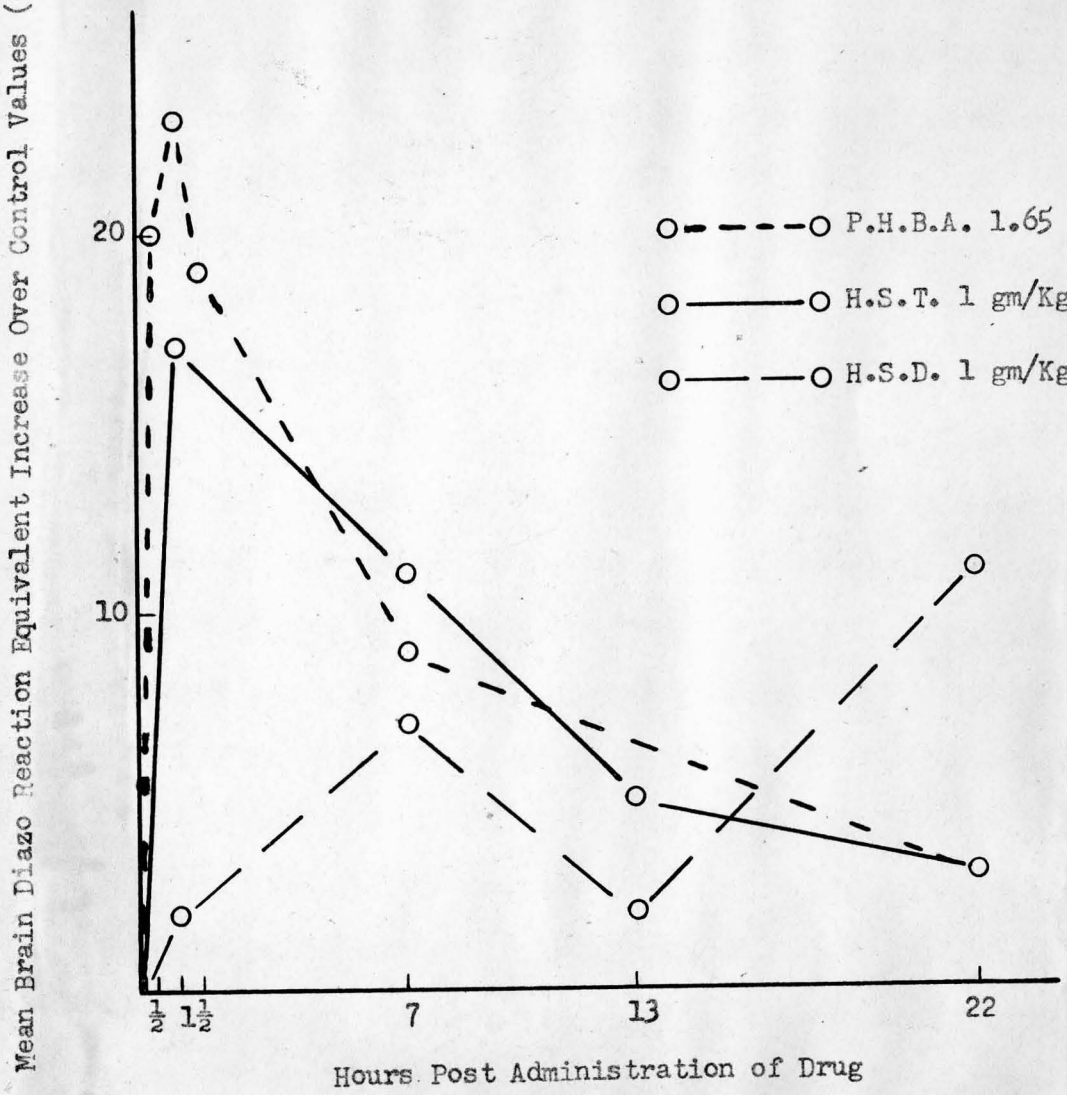

Figure 6. Comparison of Increases in Mean Brain Diazo Reaction Levels of Fasting Animals Caused by Chemotherapeutically Effective Doses of Substituted Phenols.

after administration of the drugs up to and including twenty-two hours post-drug administration. As practically all of these rises were found to be statistically significant when compared with the control means it was reasoned that a direct comparison of the drug treated mean versus the control mean differences for the three drugs was feasible. Graphic representation of this comparison is seen in figure 6. The lack of specificity of the analytical method used does not allow for the absolute conclusion that these rises in the mean brain diazo reaction equivalents are due to distribution of the administered substituted phenols in the brain. On the other hand, the conditions of the experimental method and the statistical analyses do indicate that the rises in mean brain diazo reaction equivalents are caused by administration of the drugs. That these compounds would cause a rise in the brain diazo reaction equivalent through a mechanism other than by their own distribution to that site appears to be very unlikely. This would seem to allow for the tentative conclusion that the rises observed are due to distribution of the drugs in the brain. If it is assumed that the rises in the equivalents are due to the substituted phenols administered it should also be pointed out that the methods of analysis used by the author would not necessarily determine the total amount of the compounds so distributed but only that part left free and unconjugated, if conjugation of these compounds does occur in the mouse brain. With these tentative conclusions and reservations in mind further discussion of the results can be undertaken.

Comparison of the relative activities of the three compounds against the Lansing strain of the virus of poliomyelitis with respect to the highest single rise in brain diaso reaction equivalent achieved in 22 hours with single chemotherapeutically effective doses of PHBA, HST and HSD (figure 6) seems to lead to an inverse relationship. PHBA, which exerts the least activity against the virus, yielded the highest diaso equivalent peak value (23 mg.%); HST which has more antiviral activity than PHBA but less than HSD gave a peak value of 17 mg.%, while HSD, the most potent antiviral agent of the three gave two peak values, the larger of which (11 mg.%) occurred at the twenty-second hour post-administration. These results might seem to minimise the importance of such "peak" distribution values in postulating the potential antiviral effect of a given compound. Further examination of the data will reveal that this cannot be done with safety. For example, in figure 7, where the effects of a chemotherapeutically effective dose of PHBA (1.65 mg./Kg.) on brain diaso reaction equivalents is compared with the effect of an ineffective dose (1.0 gm./kg.) on the same levels, the peak rise of the effective dose is 23 mg.% while that of the ineffective dose is 21 mg.%. As there seems to be little difference in the short time during which these peak levels are maintained it would seem to indicate, in this particular case at least, that the peak level achieved on a given day and conceivably reestablished from day to day may in some way influence the course of the infection. One other observation seems pertinent in this respect. It is interesting to note that over the same 22 hour period, HSD, the most potent antiviral compound of the three, establishes

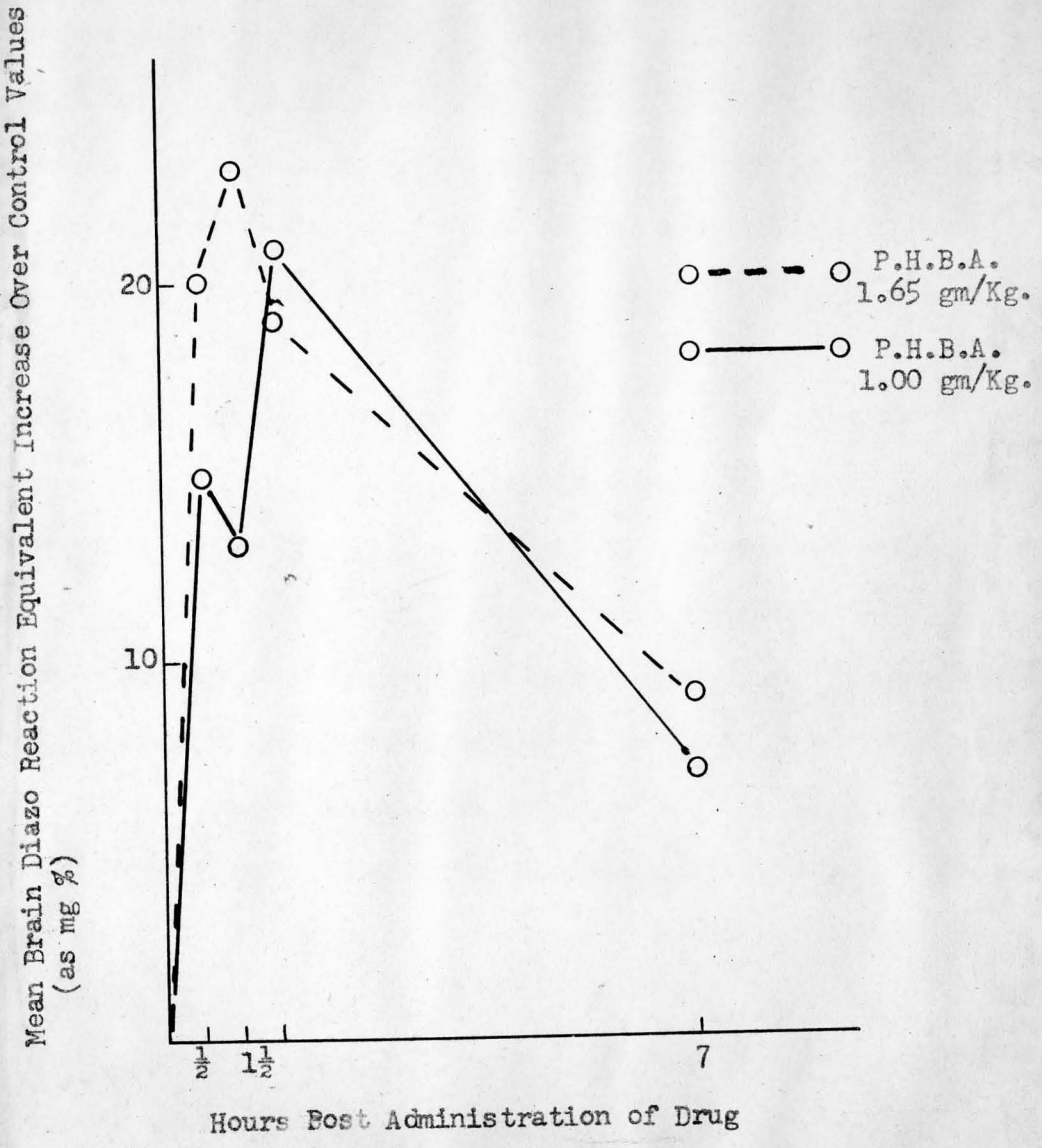

Figure 7. Comparison of Increases in Mean Brain Diazo Reaction Levels of Fasting Animals Caused by Chemotherapeutic and Sub-Chemotherapeutic Doses of P.H.B.A.

two "peak" levels, one of 7 mg.% at the seventh hour and another larger one of 11 mg.% at the twenty-second hour. After the early initial rise to peak levels within 1-1½ hours neither HST or PHBA exhibited anything other than decline in the diaso reaction levels, the decline of the latter being somewhat more precipitous than that of the former. This difference between the most potent of the three compounds and the other two in the group might conceivably mirror the existence of greater frequency of effective antiviral brain level of HSD in a given day and from day to day than is achieved by either PHBA or HST in the same unit time.

Comparison of the approximate areas of the graph (figure 6) covered by the curves of the three compounds gives a rough estimate of the over-all exposure of the brain site to the drugs in a 22 hour period. Such an approximation yields a relationship between drugs that is roughly PHBA:HSTvHSD equals 2:2:1. This data, correlated with the results obtained in the chemotherapeutic experiments, indicates that probably fewer molecules of HSD/virus/brain area are needed in order to exert antiviral activity in the case of the infection being considered. However, this interpretation must be taken with some reservation in view of the findings from experiments on non-fasting animals treated with the drugs daily.

The results of the experiments carried out on non-fasting animals given daily oral chemotherapeutically doses of the phenols are presented in figure 8. Twenty-four hours after the administration of PHBA and HST to mice slight elevations (3 mg.% and 1 mg.% respectively) over control brain diaso

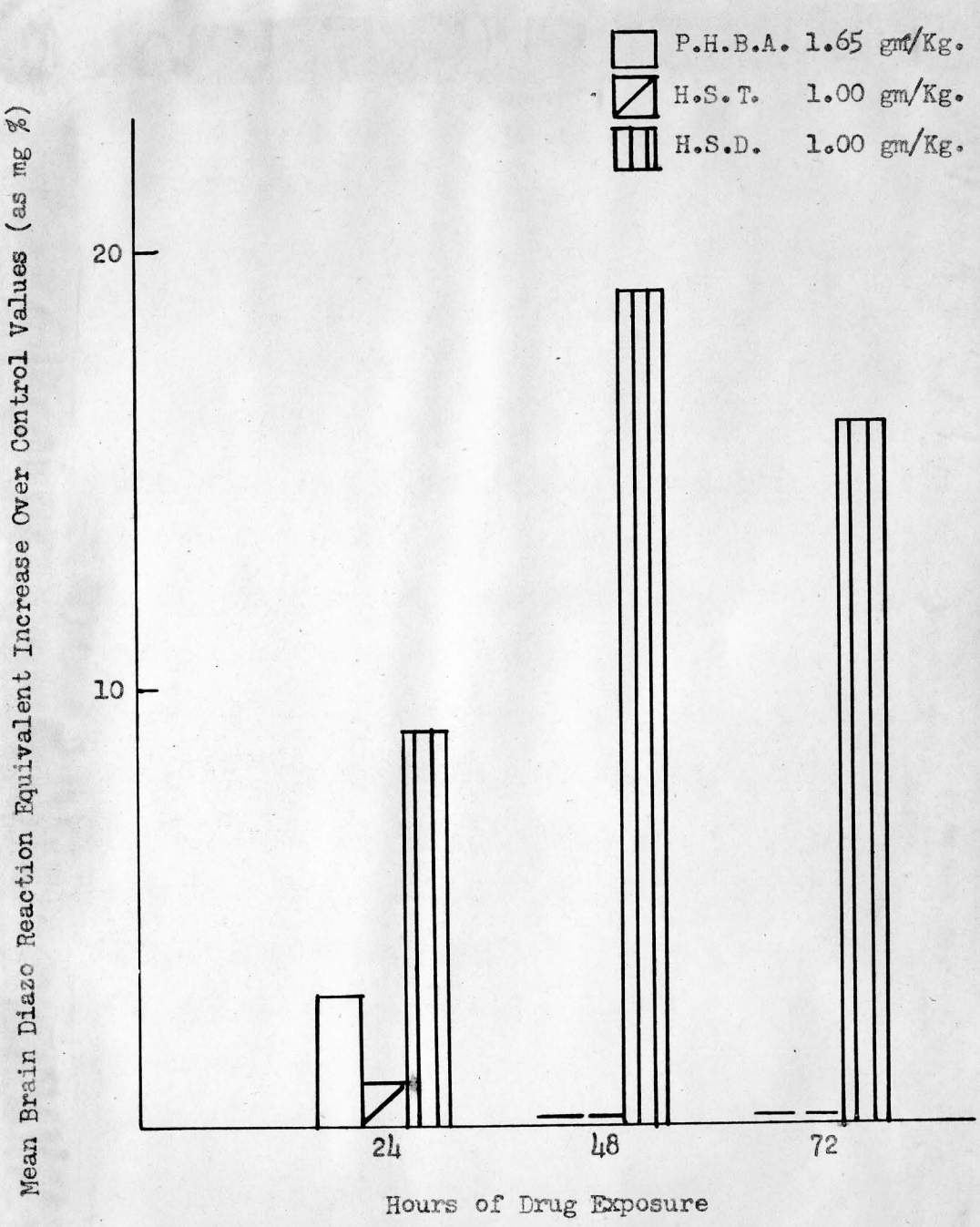

Figure 8. Comparison of Maintained Increases in Mean Brain Diazo Reaction Levels Caused by Daily Chemotherapeutically Effective Doses of Substituted Phenols.

reaction equivalents are seen. This would seem to be what is expected in view of the data obtained in the fasting animal experiments, i.e., the oral doses administered had probably caused a steep rise in the brain equivalents followed by a gradual drop to the low levels found. The twenty-four hour elevation (9 mg.%) obtained for HSD in these experiments was also not incompatible with the previous data since this could be correlated with the second peak value seen for this drug in those experiments.

However, in the case of the subgroups of animals given two doses of the respective drugs over as many days (48 hours exposure to drugs), the HSD subgroup yielded a mean brain diazo reaction elevation (19 mg.%) which was approximately double that obtained with HSD at 24 hours drug exposure, while the PHBA and HST treated animals exhibited no difference from control animals. With the data at hand it is impossible to conclude that this result is due to a true cumulative effect of HSD even though it might well be just that. Regardless of the ultimate mechanism responsible for the effect, the difference between the values for the two less potent antiviral agents (HST and PHBA) and the most potent member of the group (HSD) is striking. If fluctuation of the administered HSD to and from the brain during the twenty-four hour period following the second administration does occur it would certainly appear to fluctuate at a higher concentration level than is seen in the fluctuation over the first 24 hour period of exposure to HSD. The phenomenon seems to be somewhat stable in that a mean value of 16 mg.% elevation was obtained in the case of a third subgroup of HSD treated animals carried for 72 hours before sacrifice with daily drug administration.

CHAPTER V

SUMMARY AND CONCLUSIONS

The primary objective of this research problem was aimed at ascertaining whether or not the oral administration of three para substituted phenols para-hydroxybenzoic acid (PHBA), (N-(2-thiazolyl)-phenol sulfonamide (HST), and N-(2-pyrinidyl)-phenol sulfonamide (HSD), to mice would cause the brain diazo reaction levels of the mice to change, and, if such change was encountered, to ascertain whether or not the change could be correlated with the effects of these compounds on the course of experimental poliomyelitis infections in mice as ascertained previously by Proctor and Eyrd (1947-).

In the course of attempting to realize this objective it has been ascertained that of the two diazo reagents which have been advocated for the determination of phenolic compounds in biological material the use of diazotized sulfanilic acid for such determinations in deproteinized extracts of brain tissue is superior to the use of diazotized para-nitroaniline for the same purpose. It has also been shown that period of fasting time can significantly affect the diazo reaction levels of the brain in the mouse.

Experimental data has resulted which indicates that the oral administration of the three compounds to fasting animals brings about definite increases in the mean brain diazo reaction equivalents over the corresponding values in control mice regardless of the time elapsing after administration

of the drugs up to an including twenty-two hours post-drug administration. If, as is most likely, the elevation in the brain diaso levels observed are due to distribution of free, unconjugated phenolic derivatives in the brain, and this distribution also obtains in the virus infected animal, certain correlations between the antiviral activity of these compounds and the differences in brain distribution patterns observed are possible. The possibility that the achievement of short lived peak level concentrations of the phenolic derivatives in the brain might be associated with antiviral effect can be postulated from the results obtained in experiments where brain distribution of an effective antiviral dose of PHBA was compared with the similar effect of an ineffective dose of this compound. The peak rise achieved in the former case was only 2 mg.% greater than the peak rise demonstrated in the latter instance. As the data seem to indicate little difference in the short time during which these respective peak rises were maintained, the demonstrated effectiveness of the dose of PHBA giving the greater peak rise might be conceivably correlated with achievement of such a peak level from day to day as the drug is administered to infected animals once per day according to the procedure of Proctor and Byrd.

Maximum peak levels of concentration observed in the case of HST and HSD were lower than those found for PHBA, the HSD maximum being lower than that found for the HST. In the case of both PHBA and HST rather swift distribution of these compounds in the brain followed by steady decline in their free forms in the brain seemed to be evident. On the other hand, in the case

of HSD, relatively slow distribution of this compound in the brain seemed to take place in two phases: a slow steady distribution over a period of some five to six hours to one peak level of concentration, followed by a decline over about the same period of time with subsequent reestablishment of a second peak value after about nine hours. In this case the second peak value (11 mg.%) was greater than the first peak value (7 mg.%). This apparent ebb and flow of concentration maxima, being fundamentally different from the analagous phenomena observed in the case of the compounds with lesser potency, would present the possibility that frequency of establishment of effective peak levels of drug concentration in the brain may play a role in antiviral activity. Other data obtained from the fasting experiments would seem to indicate that in the first day of administration fewer molecules of HSD/brain area are achieved following oral administration of an effective dose than is the case with similar doses of the other compounds.

In experiments carried out on non-fasting animals given daily oral chemotherapeutically effective doses of the compound and subjected to analysis at 24 hour intervals of drug exposure time a striking difference was observed between HSD on the one hand and the other two compounds, PHBA and HST, on the other hand. The latter two compounds exhibited only slight increases in brain diaso reaction equivalents after twenty-four hour drug exposure and no rises after 48 hour drug exposure. In the case of HSD a rise approximately two times as great as that seen at 24 hours drug exposure was revealed after 48 hours drug exposure. In both instances the rises seen with HSD were significantly

greater than those elicited by the other two drugs. No increase but a fair degree of maintenance of the phenomena was seen in HSD treated animals subjected to 72 hours drug exposure. This effect of HSD cannot be ascribed to a true cumulative accumulation of HSD in the brain from the data at hand. Regardless of whether cumulative action is present or not, this data, correlated with the 22 hour experiments on the drugs, does allow for the tentative conclusion that greater frequency of peak concentration levels of this drug is achieved during the course of its administration and that the absolute level of some of these peak concentrations in the brain tends to rise after the second day of drug exposure. As this is a finding which has been observed in the case of only the most potent antiviral compound (HSD) of the three phenols under study it would seem that such activity might be associated with its greater antiviral effectiveness. It is interesting to note that the distribution of pure unsubstituted phenol in the brain of the rabbit seems to occur in such a manner as to yield peak concentration levels over periods of time similar to those studied by the author. (Deichmann, 1944). It is also possible that the greater antiviral effect of HSD is associated with the slower rise of and the more prolonged maintenance of the higher levels of this compound seen in the course of its distribution in the brain.

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APPENDIX

TABLE II

STATISTICAL EVALUATIONS PERFORMED

Means Compared	Number of Degrees of Freedom	t Value (Fischer)	Probability
1) Fasting control animals vs. fasting animals with 1/2 hour drug exposure to PHBA, 1 gm/Kg.	27	12.50	< 0.01
2) Fasting control animals vs. fasting animals with 1/2 hour drug exposure to PHBA, 1.65 gm/Kg.	28	13.00	< 0.01
3) Fasting control animals vs. fasting animals with 1 hour drug exposure to PHBA, 1 gm/Kg.	28	16.61	< 0.01
4) Fasting control animals vs. fasting animals with 1 hour drug exposure to PHBA, 1.65 gm/Kg.	28	20	< 0.01
5) Fasting control animals vs. fasting animals with 1½ hour drug exposure to PHBA, 1 gm/Kg.	22	15.49	< 0.01
6) Fasting control animals vs. fasting animals with 1½ hour drug exposure to PHBA, 1.65 gm/Kg.	22	14.73	< 0.01
7) Fasting control animals vs. fasting animals with 7 hour drug exposure to PHBA, 1 gm/Kg.	16	6.92	< 0.01

APPENDIX

TABLE II (Continued)

STATISTICAL EVALUATIONS PERFORMED

Means Compared	Number of Degrees of Freedom	t Value (Fischer)	Probability
8) Fasting control animals vs. fasting animals with 7 hour drug exposure to PHBA, 1.65 gm/Kg.	16	7.34	< 0.01
9) Fasting control animals vs. fasting animals with 22 hours drug exposure to PHBA, 1.65 gm/Kg.	22	6.2	< 0.01
10) Fasting control animals vs. fasting animals with 1 hour drug exposure to HST, 1 gm/Kg.	28	19.79	< 0.01
11) Fasting control animals vs. fasting animals with 1 hour drug exposure to HSD, 1 gm/Kg.	28	1.84	< 0.10
12) Fasting control animals vs. fasting animals with 7 hours drug exposure to HST, 1 gm/Kg.	22	12.16	< 0.01
13) Fasting control animals vs. fasting animals with 7 hours drug exposure to HSD, 1 gm/Kg.	22	7.74	< 0.01
14) Fasting control animals vs. fasting animals with 13 hours drug exposure to HST, 1 gm/Kg.	22	7.41	< 0.01
15) Fasting control animals vs. fasting animals with 13 hours drug exposure to HSD, 1 gm/Kg.	21	1.73	0.01
16) Fasting control animals vs. fasting animals with 22 hours drug exposure to HST, 1 gm/Kg.	22	3.82	< 0.01

APPENDIX

TABLE II (Continued)

STATISTICAL EVALUATIONS PERFORMED

Means Compared	Number of Degrees of Freedom	t Value (Fischer)	Probability
17) Fasting control animals vs. fasting animals with 22 hours drug exposure to HSD, 1 gm/Kg.	22	8.30	< 0.01
18) Non-fasting control animals vs. non-fasting animals with 24 hours drug exposure to HSD, 1 gm/Kg.	26	5	< 0.01
19) Non-fasting control animals vs. non-fasting animals with 48 hours drug exposure to HSD, 1 gm/Kg.	26	9.32	< 0.01
20) Non-fasting control animals vs. non-fasting animals with 72 hours drug exposure to HSD, 1 gm/Kg.	26	10.09	< 0.01
21) Non-fasting control animals vs. non-fasting animals with 24 hours drug exposure to HST, 1 gm/Kg.	26	1.09	< 0.5
22) Non-fasting control animals vs. non-fasting animals with 48 hours drug exposure to HST, 1 gm/Kg.	26	0	0.50
23) Non-fasting control animals vs. non-fasting animals with 24 hours drug exposure to PHBA, 1.65 gm/Kg.	26	4.79	< 0.01
24) Non-fasting control animals vs. non-fasting animals with 48 hours drug exposure to PHBA, 1.65 gm/Kg.	27	3	0.01

APPENDIX

TABLE II (Continued)

STATISTICAL EVALUATIONS PERFORMED

Means Compared	Number of Degrees of Freedom	t Value (Fischer)	Probability
25) Non-fasting control animals vs. fasting control animals, 3 hours (HSD, HST)	34	15.03	< 0.01
26) Fasting control animals, 3 hours vs. fasting control animals, 6 hours (HSD, HST)	22	8.12	< 0.01
27) Fasting control animals, 6 hours vs. fasting control animals, 9 hours (HSD, HST)	16	11.40	< 0.01
28) Fasting control animals, 9 hours vs. fasting control animals, 15 hours (HSD, HST)	22	16.76	< 0.01
29) Fasting control animals, 15 hours vs. fasting control animals, 18 hours (HSD, HST)	16	4.07	< 0.01
30) Fasting control animals, 18 hours vs. fasting control animals, 24 hours (HSD, HST)	16	4.38	< 0.01
31) Non-fasting control animals vs. fasting control animals, 3 hours (PHBA)	34	18.60	< 0.01
32) Fasting control animals, 3 hours vs. fasting control animals, 6 hours (PHBA)	22	8	< 0.01
33) Fasting control animals, 6 hours vs. fasting control animals, 9 hours (PHBA)	16	13.19	< 0.01
34) Fasting control animals, 9 hours vs. fasting control animals, 15 hours (PHBA)	22	20.07	< 0.01

APPENDIX

TABLE II (Continued)

STATISTICAL EVALUATIONS PERFORMED

Means Compared	Number of Degrees of Freedom	t Value (Fischer)	Probability
35) Fasting control animals, 15 hours vs. fasting control animals, 18 hours (PHBA)	16	6.08	< 0.01
36) Fasting control animals, 18 hours vs. fasting control animals, 24 hours (PHBA)	16	4.47	< 0.01
37) Fasting control animals vs. fasting animal with 1 hour exposure to gm tragacanth, 1 gm/Kg.	22	1.26	> 0.01

TABLE XII

P.H.B.A

CONTROL VALUES EXPRESSED AS DIAZO EQUIVALENTS IN mg.%

Mice Number	Non Fasting	Hours of Fasting						
		3	3 1/2	6	9	15	18	24
1	54	43	46	53	43	48	41	43
2	49	41	45	50	37	46	48	37
3	50	39	42	47	38	47	45	34
4	46	34	40	48	35	52	42	34
5	46	35	39	48	34	50	37	36
6	46	35	40	45	32	47	43	37
7	64	53	48		41	42		40
8	52	46	39		37	45		41
9	52	39	45		34	44		41
10	51	38	40		31	48		37
11	51	36	41		36	47		38
12	47	37	40		34	46		34
13	56	44						
14	46	40						
15	48	40						
16	48	40						
17	41	40						
18	50	40						
MEAN	50	40	42	48	36	47	42	38
STD. DEVIATION	± 4.96	± 4.51	± 3.08	± 2.79	± 3.61	± 2.62	± 3.79	± 3.03

TABLE IV

P.H.B.A., 1 gm./Kg., DRUG TREATED ANIMALS
VALUES EXPRESSED AS DIAZO EQUIVALENTS IN mg.%

Mice Number	Hours of Drug Exposure (Fasting)			
	1/2	1	1 1/2	2
1	58	66	76	51
2	47	58	73	48
3	45	57	54	40
4	49	53	47	44
5	53	59	61	46
6	48	52	63	36
7	53	59	75	
8	61	51	56	
9	85	51	60	
10	55	46	54	
11	46	46	59	
12		43	78	
MEAN	55	53	63	44
STD. DEVIATION	± 11.48	± 6.63	± 10.39	± 5.45

TABLE V

P.H.B.A., 1.65 gm./Kg., DRUG TREATED ANIMALS
VALUES EXPRESSED AS DIAZO EQUIVALENTS IN mg. %

Mice Number	Non-Fasting Hours of Drug Exposure		Hours of Drug Exposure (Fasting)				
	24	48	1/2	1	1 1/2	7	22
1	53	48	106	86	80	46	43
2	52	44	70	53	61	44	40
3	57	47	62	65	64	41	41
4	50	47	56	50	66	41	40
5	54	45	54	56	51	57	43
6	53	52	51	50	58	42	42
7	59	51	59	63	51		42
8	56	48	50	59	59		38
9	51	49	45	67	71		36
10	55	44	54	72	54		42
11	50	49	63	80	46		45
12	50		49	60	74		44
MEAN	53	48	60	63	61	45	42
STD. DEVIATION	± 2.98	± 2.64	± 16.06	± 11.40	± 10.19	± 6.11	± 2.54

TABLE VI

H.S.D. and H.S.T.

CONTROL VALUES EXPRESSED AS DIAZO EQUIVALENTS IN mg. %

Mice Number	Non Fasting	Hours of Fasting						
		3	3 1/2	6	9	15	18	24
1	80	64	69	78	67	71	61	68
2	71	63	69	75	58	69	74	59
3	73	61	64	70	60	70	68	53
4	67	54	62	70	57	76	64	54
5	68	55	60	73	55	73	56	57
6	68	56	62	67	51	70	66	58
7	91	79	71		62	62		61
8	76	69	59		57	67		62
9	77	59	69		54	66		66
10	75	57	61		47	70		59
11	75	56	63		57	71		58
12	69	57	60		53	70		55
13	81	67						
14	67	61						
15	71	61						
16	71	64						
17	60	62						
18	74	62						
MEAN	73	62	64	72	57	70	65	59
STD. DEVIATION	± 6.78	± 6.00	± 4.24	± 3.97	± 5.21	± 3.52	± 6.14	± 4.52

TABLE VII

H.S.D., 1 gm./Kg., DRUG TREATED ANIMALS
VALUES EXPRESSED AS DIAZO EQUIVALENTS IN mg.%

Mice Number	Non-Fasting Hours of Drug Exposure			Hours of Drug Exposure (Fasting)			
	24	48	72	1	7	13	22
1	117	151	114	85	61	82	70
2	73	111	94	76	70	73	69
3	78	85	70	60	62	78	65
4	78	77	79	58	59	66	66
5	65	86	100	58	61	74	59
6	62	78	74	54	63	85	62
7	88	92	110	68	67	72	98
8	75	82	74	70	71	59	74
9	95	89	87	59	60	78	70
10	73	84	94	55	72	66	68
11		82	72	61	60	63	68
12		80	101	63	57		72
MEAN	82	92	89	64	64	72	70
STD. DEVIATION	± 16.18	± 20.78	± 15.32	± 9.21	± 5.09	± 8.18	± 9.69

TABLE VIII

H.S.T., 1 gm./Kg., DRUG TREATED ANIMALS
VALUES EXPRESSED AS DIAZO EQUIVALENTS IN mg. %

Mice Number	Non-Fasting hours of Drug Exposure		HOURS OF DRUG EXPOSURE (FASTING)			
	24	48	1	7	13	22
1	82	83	92	71	74	70
2	80	70	74	70	72	63
3	71	73	80	66	73	62
4	69	70	73	62	72	57
5	76	75	74	64	80	63
6	75	74	70	68	76	66
7	75	74	85	80	78	68
8	82	68	81	68	70	57
9	69	71	80	71	81	58
10	77	70	80	72	70	57
11	68	71	79	64	82	58
12	65	71	79	62	75	63
MEAN	74	73	79	68	75	62
STD. DEVIATION	± 5.65	± 3.87	± 5.83	± 5.09	± 4.18	± 4.58